(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 February 2003 (13.02.2003)

PCT

(10) International Publication Number
WO 03/012112 A1

(51) International Patent Classification[7]: C12N 15/63

(21) International Application Number: PCT/SE02/01121

(22) International Filing Date: 8 July 2002 (08.07.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0102627-7 27 July 2001 (27.07.2001) SE

(71) Applicant *(for all designated States except US)*: **GENEINVENT BBL AB** [SE/SE]; Box 789, S-220 07 Lund (SE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **WIDEGREN, Bengt** [SE/SE]; Uardavägen 10 C, S-224 71 Lund (SE). **SALFORD, Leif, G.** [SE/SE]; Finngatan 9, S-220 07 Lund (SE). **PERSSON, Bertil** [SE/SE]; Angantyrs gränd 19, S-224 75 Lund (SE).

(74) Agents: **MANNERLÖF TENNING, Marie** et al.; c/o Albihns Malmö AB, P.O. Box 4289, S-203 14 Malmö (SE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/012112 A1

(54) Title: METHYLATION RESISTANT VECTORS

(57) Abstract: The invention relates to vectors produced in a donor host cell, which upon transfer into a receiver host cell maintain the desired expression of the nucleotide sequences which are located within the vector. The maintenance of the desired expression is achieved due to that the vector at least partly remains unmethylated within the receiver host cell. The donor host cell being different as compared to the receiver host cell and the receiver host cell being capable of methylating DNA. To prevent methylation, cytosines in a CpG motif in the nucleotide sequence to be transferred are replaced with cytosine analogues resistant to methylation. The invention also relates to methods for the production of such vectors and the use of the vectors in industry as well as in medicine.

METHYLATION RESISTANT VECTORS

FIELD OF INVENTION

The invention relates to vectors, which at least partly remains resistant to methylation upon transfer into a receiver host cell. The receiver host cell is different from the donor host cell. The invention also relates to methods for the production of such vectors and the use of the vectors in industry as well as in medicine.

BACKGROUND OF INVENTION

Modulation of gene expression has become an increasingly important approach to understand various cellular processes and their underlying biochemical pathways. Regulation of gene expression is a complex process, and many mechanisms of regulation remains to be understood. Methylation of DNA in eukaryotic organisms is one important mechanism of gene regulation. Methylation of DNA is a mechanism to control the expression of genes located within the genome of eukaryotic cells as well a to downregulate the expression of foreign nucleotide sequences which enters into the eukaryotic cell. The phenomenon has been investigated in both animal cells as well as in plants, and the methylation is identified to occur on the 5-position in the pyrimidine ring of cytosine located in a stretch of CpG.

Silencing of a gene or a cluster of genes may be the result of methylation of CpG motifs located in the nucleotide sequence of the gene or cluster of genes as well as methylation of regulatory sequences such as promoters and responsive elements for transcription factors. The degree of methylation influences the degree of silencing and in general a low degree of methylation result in a minor decrease in expression and a higher degree of methylation result in complete silencing of the expression of a gene.

Methylation effects the expression of polypeptides located within expression vectors. When a stretch of foreign DNA enters the nucleus of a cell, it is likely that the stretch of foreign DNA become methylated. The degree of methylation of stretch of foreign DNA will affect the level of expression from that particular DNA. The introduced DNA can also be integrated into the chromosomal DNA of a dividing cell by recombination. If that particular DNA becomes methylated prior to integration into the chromosomal DNA and cell division it will probably become methylated during subsequent cell divisions. If a high level of expression is desired then methylation of the DNA in expression vectors is a problem.

A similar problem is also found when vectors is used in medicine for

treatment of disorders and for vaccination. For example, the DNA vaccine is most often in the form of a plasmid DNA expression vector produced in bacteria and then purified and delivered to muscle or skin. DNA vaccines have been demonstrated to show efficacy against numerous viral, bacterial and parasitic disorders in animal models. However, the level of methylation of such DNA will affect the level of expression of antigenic proteins essential for the induction of immune response against the antigen. The same problem also applies to the use of DNA vectors in gene therapy.

One way to solve the methylation problem of nucleotide sequences upon transfer into a new host is by manipulation of the genes in order to remove all CpG motifs without changing the encoded polypeptides. Such modified genes will not undergo methylation. However, the strategy of using such modified genes applies well for genes with a shorter nucleotide sequence and is not applicable for larger nucleotide sequences such as vectors or when there is a need for several different vectors. Moreover, promoters used for the expression of genes are often rich in CpG motifs, which cannot be removed without impairing the function of the promoter and thereby the expression level.

There is a need for stable optimised vectors, which do not undergo methylation upon transfer into an eukaryotic cell for use in both industry as well as in medicine. By provision of such vectors, the industry will have lesser problems in obtaining constant and higher expression of genes of interest in eukaryotic cells.

Furthermore, the medicine will be provided with improved vectors that do not undergo methylation upon transfer into a receiver host cell and the expression of the nucleotide sequences located within the vectors are maintained, which is useful both in vaccination and in gene therapy as well as all in other treatments in which expression vectors are used.

BRIEF DISCLOSURE OF THE INVENTION

The invention relates to vectors which has been modified in such a way that methylation of the vector is substantially reduced upon transfer of the vector into a receiver host cell. Thereby, the expression of the nucleotide sequence of the vector within a receiver host cell is maintained at an acceptable level. A level which maintain the expected functions of the nucleotide sequences and/or the polypeptides encoded by the nucleotide sequences. The invention also relates to methods for the production of said vectors and the use of the vectors in industry as well as in pharmaceuticals, e.g., therapy and/or diagnostics.

Accordingly, in a first aspect the invention relates to a vector comprising a nucleotide sequence wherein one or two cytosines in at least one CpG motif has been replaced with a cytosine analogue resistant to methylation.

In another aspect, the invention relates to a donor host cell comprising a vector with a nucleotide sequence wherein one or two cytosines in at least one CpG motif has been replaced with a cytosine analogue resistant to methylation.

In a further aspect, the invention relates to nucleotide sequence being part of a vector obtained from a donor host cell and/or a polypeptide produced by a donor host cell.

In still a further aspect, the invention relates to a pharmaceutical composition comprising a vector and/or a donor host cell and/or a nucleotide sequence and/or a polypeptide and a pharmaceutically acceptable diluent, carrier, adjuvant or excipient.

In still a further aspect, the invention relates to a method of reducing methylation of CpG motifs in a vector in a receiver host, which method comprises replacing at least one cytosine in a CpG motif with a cytosine analogues.

In still a further aspect, the invention relates to a kit comprising a vector and/or a donor host cell.

In still a further aspect, the invention relates to a method of transferring a vector, a nucleotide sequence, a polypeptide or a pharmaceutical composition into a receiver host cell, the method being selected from the list consisting of electroporation, microprojectile bombardment and liposome mediated delivery.

In still a further aspect the invention relates to a vector, a donor host cell, a nucleotide sequence, a polypeptide or a pharmaceutical composition for use in therapy and/or in diagnostics.

In a final aspect the invention relates to the use of a vector, a donor host cell, a nucleotide sequence, a polypeptide or a pharmaceutical composition for the manufacture of a medicament for use in therapy and/or in diagnostics.

The invention provides completely novel vectors in which cytosine in CpG motifs has been replaced with a cytosine analogue. The cytosine analogue, being resistant against methylation. Said cytosine analogue replaces at least the 5-position of the pyrimidine ring of cytosine in the CpG motif. Such a replacement results in an improved vector, which upon transfer into a receiver host cell, such as an eukaryotic cell maintain the activity/expression of the nucleotide sequence of the vector. The invention further provides methods for the production of such vectors and the use of such vectors in, e.g., industry as well as in medicine.

DESCRIPTION OF THE DRAWINGS

The invention is illustrated with reference to the drawings in which

Fig 1 shows the structure of 5-azadeoxycytidine.

Fig 2 shows an example of a general structure of a cytidine derivative.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

In the context of the present application and invention the following definitions apply:

The term "nucleotide sequence" is intended to mean a sequence of two or more nucleotides. The nucleotides may be of genomic DNA, cDNA, RNA, semisynthetic or synthetic origin or a mixture thereof. The term includes circular, linear, single and double stranded forms of DNA or RNA.

The term "vector" is intended to mean a nucleotide sequence, usually being a circular duplex of DNA having the ability to multiplicate independently of chromosomal DNA into numbers of copies in a host, i.e., having a start of replication. The vector may also be a vector, which integrate into the genome of the receiver host cell. Furthermore the vector is stably maintained and/or produced in the host during the multiplication, such as by the use of a selectable marker in the vector. The vector may be a bacteriophage, plasmid, phagemid, viral vector, plant transformation vector, insect vector or yeast artificial chromosome. Additionally, the vector may be a nucleotide sequence useful as antisense or to form aptameres.

The term "start of replication" is intended to mean a nucleotide sequence at, which DNA synthesis for replication of the vector begins. Start of replication may occur at one or more points within the vector dependent on the vector being used, such as at one point in a plasmid vector or at several points in an adenovector. The start of replication is generally termed origin of replication (abbreviated ori site) in a plasmid vector.

The term "selectable marker" is intended to mean, e.g., a gene encoding a polypeptide which confers resistance to a drug, e.g., ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate.

The term "control sequence" or "control sequences" is intended to mean nucleotide sequences involved in control of a response of action. This includes nucleotide sequences and/or proteins involved in regulating, controlling or affecting the expression of structural genes, or the replication, selection or maintenance of a plasmid or a viral vector. Examples include attenuators, silencers, enhancers, operators, terminators and promoters.

The term "CpG motif" is intended to mean a double stranded nucleotide sequence having a cytosine followed by a guanine linked by a phosphate bond, i.e., the CpG motif has two cytosines, one located in each strand of the double stranded nucleotide sequence.

The term "methylation" or "methylated CpG motif" is intended to mean that cytosine is methylated on the pyrimidine ring, usually occuring at the 5-position of

the pyrimidine ring. One or more methylated CpG motifs in a nucleotide sequence might result in complete silencing of the expression of the nucleotide sequence. If the methylated CpG motifs are located within a nucleotide sequence encoding a polypeptide or within other nucleotide sequences involved in the expression of a specific nucleotide sequence, e.g., a control sequence as defined above substantial or complete reduction of the expression of said nucleotide sequence is obtained.

The term "resistant against methylation" is intended to mean absence of methylation of one or both of the 5-position on the pyrimidine rings located within the CpG motif. One or more CpG-motifs may be unmethylated within a vector resistant against methylation. The resistance against methylation is achieved by replacement of an existing cytosine with a cytosine analogue.

The term "receiver host cell" is intended to mean a cell, which is different compared to the donor host cell. Inside a receiver host cell, a vector becomes methylated at the CpG motifs since the receiver host cell initiate methylation of CpG motifs at nucleotide sequences which are different as compared to the nucleotide sequence of the receiver host cell. This may occur when the receiver host cell, receive a vector which has been produced/multiplied in a donor host cell. The methylation of the vector result in a reduced or complete silencing of the vector.

The term "replaced with a cytosine analogue" is intended to mean an existing cytosine in a CpG motif as defined above being replaced by a cytosine analogue. The cytosine analogue replaces at least the 5-position of the pyrimidine ring of cytosine. The cytosine analogue, being resistant against methylation. However, the cytosine analogue may replace cytosine partly or completely. Cytosine is replaced with a cytosine analogue during replication of the vector.

The term "cytidine derivative" is intended to mean a cytidine derivative able to replace cytosine in a CpG motif without influencing the activity of the nucleotide sequence harbouring the replaced cytidine derivative, e.g., the activity of the nucleotide sequence is the same as prior replacement of cytosine with a cytidine derivative. For example a nucleotide sequence encoding a polypeptide will after replacement of one or more cytosines with cytidine derivatives still be active and express the polypeptide. The cytidine derivative is a derivative, which is unable to undergo methylation at the 5-position of the pyrimidine ring present in the cytidine derivative. Examples of cytidine derivatives are found in Fig 1 and 2.

The term "maintain expression" is intended to mean expression of the nucleotide sequence encoding a polypeptide and harbouring one or more cytosine analogues, i.e, maintain the expression to at least an acceptable level. The level being higher than the unmethylated form of the nucleotide sequence. The maintained expression may be within a range, which is higher than the expression of the methylated form of the same nucleotide sequence or the expression may be as

high as the expression of the unmethylated form of the same nucleotide sequence.

The term "donor host cell" is intended to mean a cell used for the production/multiplication of the vector. During growth or multiplication of the donor host cell, the copy number of the vector increase and at the same time cytosine in CpG motifs is replaced with a cytidine analogue, when the cytosine analogue is added in a sufficient amount to the growth medium used for growth of the donor host cell. The donor host cell is different as compared to the receiver host cell, such as the donor cell being a bacterial cell or a viral particle and the receiver host cell being an animal or a plant cell.

Vector of the invention.

The vector according to the invention is a vector comprising a nucleotide sequence in which one or two cytosines in at least one CpG motif has been replaced with a cytosine analogue, the cytosine analogue being resistant against methylation.

By providing said vector, which is resistant against methylation, the expression of the nucleotide sequences located within the vector is maintained to a level, which is higher as compared to the methylated form of the vector. Thereby, the function of the vector is maintained. Furthermore, replacement of cytosine with cytosine analogues may be used to control the expression of nucleotide sequences. Reducing or increasing the level of methylation result in different levels of expression, such that an increased level of methylation, result in a decrease of the level of expression. By replacing cytosine with a cytosine analogue it is possible to avoid methylation problems, which may arise when the vector is introduced into a receiver host cell.

According to a first embodiment cytosine is replaced with a cytosine analogue which replaces the 5-position in the pyrimidine ring of cytosine located in the CpG motif. The cytosine analogue may be N, O or C-X. X in C-X may be a low or non-electrophilic group, such as ethyl or methoxy. Furthermore the cytosine may be replaced by a cytidine derivative, such as 5-azacytidine and/or 5-azadeoxycytidine. 5-azadeoxycytidine may also be named s-Triazin-2(1H)-one, 4-amino-1-(2-deoxy-.beta.-D-erythro-pentofuranosyl- (8CI), 1,3, 5-Triazin-2(1H)-one, 4-amino-1-(2-deoxy-.beta.-D-erythro-pentofuranosyl)- (9CI), 5-Aza-2'-deoxycytidine or Decitabine (USAN).

The number and the distribution of the cytosin analogues within CpG motifs of the vector inhibits methylation of the 5-position of the pyrimidine ring resulting in maintenance of the expression of the nucleotide sequence encoding polypeptides located within the vector. An increased number of integrated cytosine analogous within a nucleotide sequence region encoding a polypeptide of the vector result in

an increased possibility of maintaining the expression of the nucleotide sequences present in that region of nucleotide sequences. Increased number of integrated cytosine analogous within other regions of the vector may indirectly influence the maintenance of a nucleotide sequence region encoding a polypeptide. Examples of nucleotide sequence regions, which indirectly influence other nucleotide sequence regions, are control sequences such as promoters.

A CpG motif includes two cytosines having the ability of being replaced by a cytosine analogue. Either one or both of them may be replaced. In the case several cytosine analogues are integrated into the vector. The cytosine analogues may either be integrated in one and the same strand or both strands. The cytosine analogues are integrated into the vector during replication of that vector.

The number of cytosine analogues and the their distribution result in resistance against methylation of said cytosine analogues. The presence of said cytosine analogues maintain the expression of nucleotide sequences including cytosine analogues to an acceptable level.

In one embodiment at least 1% (e.g. 1-5%), of the CpG motifs are replaced with cytosine analogues, such as 5% (e.g. 5-10%), 10% (e.g. 10-20%), 20% (e.g.20-30%), 30% (e.g. 30-40%), 40% (e.g. 40-50%), 50% (e.g. 50-60%). Preferably from about 1 to about 100% of the cytosines are replaced by cytosine analogues, such as from 1 to about 95% or from about 5 to about 95%. The cytosine analogues being integrated either in one or in both strands of the nucleotide sequence of the vector.

The vector may comprise at least one gene or part of one gene. Which gene depends on what the vector will be used for. A potential gene candidate may be one which give rise to an immune response against influenza and useful for the purpose of vaccination of human beings. Furthermore the vector may comprise one or more control sequences. Control sequences, which enable the possibility to maintain and multiplicate the vector within a suitable donor host and/or control sequences which enable the possibility to maintain and/or multiplicate the vector within the receiver host cell. Additional control sequences may be present, which enable the possibility to express a gene and/or part of a gene, within a receiver host cell at a suitable level of expression. The choice of control sequences is dependent on the desired level of expression of the gene or part of the gene and a person skilled in the art will be able to identify such control sequences.

The vector may be a nucleotide sequence as defined above which may also be used therapeutically as anti-sense DNA. Concerns are taken in the production of such DNA and CpG di-nucleotides are omitted, if possible, in the contructs. Antisense DNA is used, for example, to interfere with the nuclear DNA and triple helix motifs are formed. Such triple helix structures are impaired in transcriptional activity or the interfering DNA can be coupled to strand breaking activities. Anti

sense DNA can also be used to bind mRNA in such a way so that the translation of the mRNA is impaired and so that RNase H activity is induced and the mRNA is degraded.

The vector may be a nucleotide sequence as defined above, which may form aptameres. Aptameres are rather short linear DNA, that binds in a specific manner to different biological molecules such as specific protein motifs, carbohydrates, and steroids. The aptameres can thereby block the activity of certain biological molecules.

The vector may be any vector as long as the vector is capable to multiply within a host (receiver and/or donor), i.e, having a start of replication. It should be understood that not all vectors and control sequences function equally well to express a nucleotide sequence of interest. Neither will a host function equally well with the same expression system. However, a person skilled in the art will be able to make a selection among these vectors, control sequences and hosts without undue experimentation. For example, in selecting a vector, the host must be considered because the vector must replicate in the donor and/or the receiver host cell or be able to integrate into the chromosome of the receiver host cell. The vector's copy number, the ability to control the copy number, and the expression of any other proteins encoded by the vector, such as selectable markers, should also be considered. In selecting a control sequence, a variety of factors should be considered. These include, for example, the relative strength of the sequence, its controllability, and its compatibility with the nucleotide sequence encoding the polypeptide of interest, particularly as regards potential secondary structures. Hosts should be selected by consideration of their compatibility with the chosen vector, the toxicity of the polypeptide encoded by the nucleotide sequence, their secretion characters, their ability to fold the polypeptide correctly, their fermentation or culture requirements, and ease of purification of the products encoded by the nucleotide sequence.

The vector may be an autonomously replicating vector, i.e, a vector, which exists as an extra chromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid. Alternatively, the vector is one which, integrate into the receiver host cell genome and replicate together with the chromosome(s) into which it has been integrated. A vector which integrate into the genome of the receiver host cell may be advantageous to use, when a certain gene and/or part of a gene and/or control sequence are in the need to be protected against future possible methylation problems. Methylation might occur upon replication of the genome into which the vector has been integrated. Another example is when there is a need of removing a certain gene and/or part of a gene and/or control sequence present in a genome. The expression of the gene and/or part of the gene

and/or the control sequence may be reduced due to methylation and replacement of such a methylated gene and/or part of a gene and/or control sequence with an unmethylated form may increase the pre-existing expression level. The replacement may be performed using methods such as homologous recombination using linear DNA. The gene and/or part of a gene and/or control sequence may by such replacements be up or down regulated. A vector comprising one or more cytidine analogues which is integrated into a genome may upon replication of the genome give rise to new copies of the genome comprising said vector without one or more cytidine analogous. However, the new copy of the genome harbouring a vector may anyway be resistant against methylation even if no cytidine analogue is present within the new copy of the vector located in the new copy of the genome.

The vector may be an expression vector, in which the nucleotide sequence encoding the polypeptide of the invention is operably linked to additional segments required for transcription of the nucleotide sequence. The vector is typically derived from plasmid or viral DNA. A number of suitable expression vectors for expression in the host cells mentioned herein are commercially available or described in the literature. Useful expression vectors for eukaryotic hosts, include, for example, vectors comprising control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Specific vectors are, e.g., pCDNA3.1(+)\Hyg (Invitrogen, Carlsbad, CA, USA) and pCI-neo (Stratagene, La Jolla, CA, USA). Useful expression vectors for yeast cells include the 2μ plasmid and derivatives thereof, the POT1 vector (US 4,931,373), the pJSO37 vector described in Okkels, Ann. New York Acad. Sci. 782, 202-207, 1996, and pPICZ A, B or C (Invitrogen). Useful vectors for insect cells include pVL941, pBG311 (Cate *et al.*, "Isolation of the Bovine and Human Genes for Mullerian Inhibiting Substance and Expression of the Human Gene in Animal Cells", Cell, 45, pp. 685-98 (1986), pBluebac 4.5 and pMelbac (both available from Invitrogen). Useful expression vectors for bacterial hosts include known bacterial plasmids, such as plasmids from *E. coli*, including pBR322, pET3a and pET12a (both from Novagen Inc., WI, USA), wider host range plasmids, such as RP4, phage DNAs, e.g., the numerous derivatives of phage lambda, e.g., NM989, and other DNA phages, such as M13 and filamentous single stranded DNA phages. Examples of suitable viral vectors are Adenoviral vectors, Adeno associated viral vectors, retroviral vectors, lentiviral vectors, herpes vectors and cytomegalo viral vectors.

Other vectors for use in this invention include those that allow the nucleotide sequence encoding the polypeptide to be amplified in copy number. Such amplifiable vectors are well known in the art. They include, for example, vectors able to be amplified by DHFR amplification (see, e.g., Kaufman, U.S. Pat. No. 4,470,461, Kaufman and Sharp, "Construction Of A Modular Dihydrafolate

Reductase cDNA Gene: Analysis Of Signals Utilized For Efficient Expression", Mol. Cell. Biol., 2, pp. 1304-19 (1982)) and glutamine synthetase ("GS") amplification (see, e.g., US 5,122,464 and EP 338,841).

The recombinant vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV40 start of replication. When the host cell is a yeast cell, suitable sequences enabling the vector to replicate are the yeast plasmid 2μ replication genes REP 1-3 and start of replication.

The vector may also comprise a selectable marker, e.g., a gene the product of which complements a toxin related deficiency in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the Schizosaccharomyces pombe TPI gene (described by P.R. Russell, Gene 40, 1985, pp. 125-130), or one which confers resistance to a drug, e.g., ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate. For *Saccharomyces cerevisiae*, selectable markers include *ura3 and leu2*. For filamentous fungi, selectable markers include *amdS*, *pyrG*, *arcB*, *niaD* and *sC*.

The term "control sequences" is defined herein to include all components, which are necessary or advantageous for the expression of the polypeptide of the invention. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide. Such control sequences include, but are not limited to, a leader sequence, polyadenylation sequence, propeptide sequence, promoter (inducible or constitutive), enhancer or upstream activating sequence, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter.

A wide variety of control sequences may be used in the present invention. Such useful control sequences include the control sequences associated with structural genes of the foregoing expression vectors as well as any sequence known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

Examples of suitable control sequences for directing transcription in mammalian cells include the early and late promoters of SV40 and adenovirus, e.g. the adenovirus 2 major late promoter, the MT-1 (metallothionein gene) promoter, the human or rodent cytomegalovirus immediate-early gene promoter (CMV), the human elongation factor 1α (EF-1α) promoter, the *Drosophila* minimal heat shock protein 70 promoter, the Rous Sarcoma Virus (RSV) promoter, the human ubiquitin C (UbC) promoter, the human growth hormone terminator, SV40 or adenovirus Elb region polyadenylation signals and the Kozak consensus sequence (Kozak, M. *J Mol Biol* 1987 Aug 20;196(4):947-50).

In order to improve expression in mammalian cells a synthetic intron may be

inserted in the 5' untranslated region of the nucleotide sequence encoding the polypeptide. An example of a synthetic intron is the synthetic intron from the plasmid pCI-Neo or from the β-globin gene (available from Promega Corporation, WI, USA). Moreover an IRES element might be included.

Examples of suitable control sequences for directing transcription in insect cells include the polyhedrin promoter, the P10 promoter, the *Autographa californica* polyhedrosis virus basic protein promoter, the baculovirus immediate early gene 1 promoter and the baculovirus 39K delayed-early gene promoter, and the SV40 polyadenylation sequence. Examples of suitable control sequences for use in yeast host cells include the promoters of the yeast α-mating system, the yeast triose phosphate isomerase (TPI) promoter, promoters from yeast glycolytic genes or alcohol dehydrogenase genes, the ADH2-4c promoter, and the inducible GAL promoter. Examples of suitable control sequences for use in filamentous fungal host cells include the ADH3 promoter and terminator, a promoter derived from the genes encoding *Aspergillus oryzae* TAKA amylase triose phosphate isomerase or alkaline protease, an *A. niger* α-amylase, *A. niger* or *A. nidulans* glucoamylase, *A. nidulans* acetamidase, *Rhizomucor miehei* aspartic proteinase or lipase, the TPI1 terminator and the ADH3 terminator. Examples of suitable control sequences for use in bacterial host cells include promoters of the *lac* system, the *trp* system, the *TAC* or *TRC* system, and the major promoter regions of phage lambda.

The presence or absence of a signal peptide will, e.g., depend on the expression host cell used for the production of the polypeptide to be expressed (whether it is an intracellular or extracellular polypeptide) and whether it is desirable to obtain secretion. For use in filamentous fungi, the signal peptide may conveniently be derived from a gene encoding an *Aspergillus* sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or protease or a *Humicola lanuginosa* lipase. The signal peptide is preferably derived from a gene encoding *A. oryzae* TAKA amylase, *A. niger* neutral α-amylase, *A. niger* acid-stable amylase, or *A. niger* glucoamylase. For use in insect cells, the signal peptide may conveniently be derived from an insect gene (cf. WO 90/05783), such as the *Lepidopteran manduca sexta* adipokinetic hormone precursor, (cf. US 5,023,328), the honeybee melittin (Invitrogen), ecdysteroid UDPglucosyltransferase (egt) (Murphy *et al.*, Protein Expression and Purification 4, 349-357 (1993) or human pancreatic lipase (hpl) (Methods in Enzymology 284, pp. 262-272, 1997). A preferred signal peptide for use in mammalian cells is that of hG-CSF or the murine Ig kappa light chain signal peptide (Coloma, M (1992) J. Imm. Methods 152:89-104). For use in yeast cells suitable signal peptides have been found to be the α-factor signal peptide from *S. cereviciae* (cf. US 4,870,008), a modified carboxypeptidase signal peptide (cf. L.A. Valls *et al.*, Cell 48, 1987, pp. 887-897),

the yeast BAR1 signal peptide (cf. WO 87/02670), the yeast aspartic protease 3 (YAP3) signal peptide (cf. M. Egel-Mitani *et al.*, Yeast 6, 1990, pp. 127-137), and the synthetic leader sequence TA57 (WO98/32867).

Any suitable donor host cell may be used for the maintenance and production of the vector of the invention, such as an eukaryotic or procaryotic cell, for example bacteria, fungi (including yeast), plant, insect, mammal, or other appropriate animal cells or cell lines, as well as transgenic animals or plants. The donor host cell may be a donor host cell belonging to a GMP (Good Manufacturing Practice) certified cell-line, such as a mammalian cell-line. Examples of bacterial donor host cells include grampositive bacteria such as strains of *Bacillus*, e.g. *B. brevis* or *B. subtilis*, *Pseudomonas* or *Streptomyces*, or gramnegative bacteria, such as strains of *E. coli*. Examples of suitable filamentous fungal donor host cells include strains of *Aspergillus*, e.g. *A. oryzae*, *A. niger*, or *A. nidulans*, *Fusarium* or *Trichoderma*. Examples of suitable yeast donor host cells include strains of *Saccharomyces*, e.g. *S. cerevisiae*, *Schizosaccharomyces*, *Klyveromyces*, *Pichia*, such as *P. pastoris* or *P. methanolica*, *Hansenula*, such as *H. Polymorpha* or *Yarrowia*. Examples of suitable insect donor host cells include a *Lepidoptora* cell line, such as *Spodoptera frugiperda* (Sf9 or Sf21) or *Trichoplusioa ni* cells (High Five) (US 5,077,214). Examples of suitable mammalian donor host cells include Chinese hamster ovary (CHO) cell lines, (e.g. CHO-K1; ATCC CCL-61), Green Monkey cell lines (COS) (e.g. COS 1 (ATCC CRL-1650), COS 7 (ATCC CRL-1651)); mouse cells (e.g. NS/O), Baby Hamster Kidney (BHK) cell lines (e.g. ATCC CRL-1632 or ATCC CCL-10), and human cells (e.g. HEK 293 (ATCC CRL-1573)), as well as plant cells in tissue culture. Additional suitable donor cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Rockville, Maryland.

In a specific embodiment the invention relates to a nucleotide sequence obtained from a donor host harbouring the nucleotide sequence being part of the vector. The vector and the donor host are a vector and a donor host as described above. The nucleotide sequence may be a transcriptional unit comprising a promoter a nucleotide sequence, such as a gene or part of a gene and polyadenylation site. The nucleotide sequence may be a gene and/or part of a gene and/or one or more control sequences as mentioned above. One example of gene or genes of interest are genes encoding nuclear core proteins, such as the influenza nuclear core protein encoding genes which may be used for vaccination. Another example is the gene encoding tyrosin hydroxylase, which may be useful for the treatment of Parkinsons disease. A third example is genes encoding factor X or VIII involved in the blood clotting complex or interferon gamma. Additionally, all kind of genes useful in gene therapy such as treatment of cancer are gene candidates useful to be used according to the

invention, such as thymidin kinase from Herpes Simplex Virus (HSV). The nucleotide sequence may be used as antisense. Antisense DNA is used, for example, to interfere with the nuclear DNA so that triple helix motifs are formed. Such triple helix structures are impaired in transcriptional activity or the interfering DNA can be coupled to strand breaking activities. Anti sense DNA can also be used to bind mRNA in such a way so that the translation of the mRNA is impaired and so that RNase H activity is induced and the mRNA is degraded. Additionally, the nucleotide sequence may be used as aptameres, which is short stretches of nucleotide sequences that binds in a specific manner to different biological molecules such as specific protein motifs, carbohydrates, steroids etc. The use of a cytosine base analogue, such as aza-deoxy-cytidine, that cannot be methylated, can lower the immunogenicity of unmethylated DNA and thereby reduce the immunostimulatory side effects. The vector and/or the nucleotide sequence such as transcriptional units amplified by PCR may be used to transfect cells. The vector may be linearized and, e.g., prokaryotic parts can be left out by digestion with restriction enzymes. Such, in vitro produced, DNA can be used for, e.g. for immunization or in gene therapy, ex vivo or in vivo.

In another embodiment the invention relates to a polypeptide produced by the donor host cell, the polypeptide is encoded by a nucleotide sequence being part of the vector according to the invention and described above.

In another embodiment the invention relates to a pharmaceutical composition comprising the above described vector and/or the donor host and/or a nucleotide sequence obtained from the vector and/or a polypeptide and a pharmaceutically acceptable diluent, carrier, adjuvant or excipient.

The vector, nucleotide sequence, polypeptide or a pharmaceutical composition as described above may be transferred into a receiver host cell by the use of a suitable transformation method such as electroporation, microprojectile bombardment or liposome mediated delivery.

The vector, nucleotide sequence, polypeptide or a pharmaceutical composition as described above may be used in therapy and/or in diagnostic.

The vector, nucleotide sequence, polypeptide or a pharmaceutical composition as described above may be used for the manufacture of a medicament for use in therapy and/or in diagnostic.

Method for the production of the vector of the invention.

One embodiment of the invention relates to a method, which reduce methylation of CpG motifs in a vector in a receiver host, which method comprises replacing at least one cytosine in a CpG motif with a cytosine analogous, the vector being described above. The method may be a cultivation method or a method, which

amplifies the vector, such as by PCR.

According to another embodiment according to the invention, the method comprising the steps of; providing a vector; transferring the vector into a donor host cell; growing the donor host cell harbouring the vector in a growth medium consisting of a suitable amount of cytosine analogous and harvesting the multiplied vector comprising of incorporated cytosine analogous in one or two cytosines in at least one one CpG motifs. The vector being a vector as described above and the donor host being a donor host as described above.

The vector and the donor host is chosen dependent upon the purpose of creating that specific vector with incorporated cytosine analogous and a person skilled in the art may perform that selection. The vector is transferred (introduced) into the donor host using a suitable method dependent on which donor host has been selected. The introduction of a vector into a bacterial donor host cell may, for instance, be effected by protoplast transformation (see, *e.g.*, Chang and Cohen, 1979, *Molecular General Genetics* 168: 111-115), using competent cells (see, *e.g.*, Young and Spizizin, 1961, *Journal of Bacteriology* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56: 209-221), electroporation (see, *e.g.*, Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or conjugation (see, *e.g.*, Koehler and Thorne, 1987, *Journal of Bacteriology* 169: 5771-5278). Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* host cells are described in EP 238 023 and US 5,679,543. Suitable methods for transforming *Fusarium* species are described by Malardier *et al.*, 1989, *Gene* 78: 147-156 and WO 96/00787. Yeast may be transformed using the procedures described by Becker and Guarente, *In* Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito *et al.*, 1983, *Journal of Bacteriology* 153: 163; Hinnen *et al.*, 1978, *Proceedings of the National Academy of Sciences USA* 75: 1920: and as disclosed by Clontech Laboratories, Inc, Palo Alto, CA, USA (in the product protocol for the Yeastmaker™ Yeast Transformation System Kit). Transformation of insect cells and production of heterologous polypeptides therein may be performed as described by Invitrogen. Methods for introducing exogeneous DNA into mammalian donor host cells include calcium phosphate-mediated transfection, electroporation, DEAE-dextran mediated transfection, liposome-mediated transfection, viral vectors and the transfection method described by Life Technologies Ltd, Paisley, UK using Lipofectamin 2000. These methods are well known in the art and e.g., described by Ausbel *et al.* (eds.), 1996, Current Protocols in Molecular Biology, John Wiley & Sons, New York, USA.

In the production methods of the present invention, the cells are cultivated in a growth medium suitable for maintenance and/or production of the vector using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermenters performed in a suitable medium and under conditions allowing the vector, nucleotide sequence or polypeptide to be expressed and/or isolated. The vector, nucleotide sequence or the polypeptide may be used in the chemical or in the pharmaceutical industry. The cultivation takes place in a suitable growth medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (*e.g.*, in catalogues of the American Type Culture Collection). The cultivation of mammalian donor cells are conducted according to established methods, e.g., as disclosed in (Animal Cell Biotechnology, Methods and Protocols, Edited by Nigel Jenkins, 1999, Human Press Inc, Totowa, New Jersey, USA and Harrison MA and Rae IF, General Techniques of Cell Culture, Cambridge University Press 1997).

The cytosine analogue as described above are added to the growth medium in a suitable amount, which is sufficient to obtain a vector having a sufficiently amount of cytosine analogous incorporated into the nucleotide sequence to yield a desired the expression the nucleotide sequences encoding polypeptides. The amount of cytosine analogue is dependent on several factors including the size of the vector, the amount of CpG motifs and the host cell. For example in eukaryotic cells often 10μM of 5-azacytidine and/or 5-azadeoxycytidine is used. The cytosine analogue may be added either prior to cultivation or during the cultivation step.

After cultivation the donor host is harvested by for example centrifugation and the vector recovered by methods known in the art. Examples of methods are those mentioned in Maniatis et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbour Press) (1989) and Qiagen Inc..

The amount and distribution of cytosine analogous present in the vector after production in a suitable donor host may be determined by for example using labelled 5-azacytidine and/or 5-azadeoxycytidine, HPLC, GC or TC.

Additionally, the vector as defined above may be produced by amplification, such as by the use of PCR. One example is the amplification of a transcriptional unit by PCR and the use of such units to transfect cells directly. Another possibility is to use the vector of the invention, such as a plasmid vector for transfection. The vector may be linearized and, e.g., prokaryotic parts can be left out by digestion with restriction enzymes. By the use of PCR all sites, which may be replaced by a cytosine analogue can be replaced due to the absence of dCTP during the

amplification reaction. The amplified vector may be used for immunisation or in gene therapy, ex vivo or in vivo. One example is transfection of a mammalian cell line with a transcriptional unit, including a promoter, a cDNA and a polyadenylation site, which may be done to produce a protein encoded by the cDNA that will be secreted from the cells in a fermentor.

Kit of the invention.

Accordingly the invention relates to a kit comprising a vector as described above and/or a donor host cell as described above including a description how to use the vector and/or the vector and the donor host cell.

The kit may further comprise a growth medium suitable for the donor host cell for the ability to grow (multiply) the vector.

The kit may be used for the production of a polypeptide in industry or in a method for the treatment of a mammal suffering from a disorder or in need of vaccination. Examples of disorders are influenza and cancer.

Pharmaceutical use and formulations

According to one embodiment of the invention the vector, donor host, nucleotide sequence, polypeptide or the pharmaceutical composition according to the invention may be used for several applications including vaccination and gene therapy.

However, when the vector and/or the donor host is used for the treatment of a disorder or vaccination it is important that the nucleotide sequences creating the vector are nucleotide sequences (e.g., start of replication and control sequences) which maintain their activity such as express a polypeptide within the reciever host, i.e. the cells of the patient. For example in the case when the vector is used for vaccination of a human, it is important that a promoter is used upstream of the nucleotide sequence encoding a polypeptide that give rise to the immune response, i.e, the promoter is active in a human cell. The promoter may be inducible and/or constitutive. In the case of using an inducible promoter it may be inducible by an agent either administered together or subsequently with the vector and/ or the donor host. The promoter may further be induced by an agent produced within the patient either locally or systemically.

For example, if plasmids encoding insulin is introduced intramusculary by electroportation, then this plasmid will express insulin for a while and thereafter the expression diminishes. A methylation resistant plasmid, on the other hand, will probably have a constant expression for a long period of time. If such a plasmid have a tetracycline-inducible promoter the insulin production could be controlled by oral administration of tetracycline. This type of gene therapy could be applicable for

patients with insulin deficient type diabetis or if the gene to be expressed is a missing coagulation factor by hemophilic patients.

In a second embodiment the vector/donor host, nucleotide sequence, polypeptide or the pharmaceutical composition according to the invention is used for the manufacture of a medicament for treatment of disorders or vaccination, such as cancer. In cancer therapy of today there is a focus on therapies other than the classical radiotherapy and chemotherapy. Anti-angiogenic therapy as well as immunotherapy against cancer being two promising fields. Both therapies can utilise gene therapy for the production of anti-angiogenic agents or for the production of immunostimulatory or toxic agents. These types of therapy can be optimised by the use methylation resistants vectors, both as pure plasmids or as viral vectors made resistant against methylation. For example, a genetically modified adenovirus which express the gene for Herpes Simplex Thymidine kinase (HSV-TK) may be produced in the presence of a described cytosine analogue. Then the DNA of the adenovirus will contain this analogue and upon infection the adenovirus will express higher levels of HSV-TK for a longer period of time compared to an adenovirus not having methylation resistant DNA. The tumor cells can then be killed by a herpes virus drug. The killing is correlated with the production of HSV-TK, i.e., the higher the concentration of HSV-TK the more sensitive will the tumor cell be for the drug.

In another embodiment the vector/donor host, nucleotide sequence, polypeptide or the pharmaceutical composition according to the invention is used in a method for treating a mammal having a disorder or in need of vaccination. Vaccination has traditionally been performed by the use of mutated or killed pathogenic viruses. DNA vaccination makes use of proteins encoded by the DNA of the pathogen. Such a gene or genes are cloned in a plasmid or another vector. Plasmids encoding pathogenic protein(s) can be injected, e.g., intramusculary. The plasmid will express the pathogenic protein(s) that are recognised by the immune system of that individual. This type of immunisation then protects against subsequent infection of the pathogen. The strength of the immune reaction depends on the production of the pathogenic protein. A too low production of the pathogenic protein(s) might not be recognised by the immune system. Hence, immunisation of both humans and animals with methylation resistant vectors will improve the efficiency.

Pharmaceutical formulations of the vector/donor host, nucleotide sequence, polypeptide of the invention are typically administered in a composition that includes one or more pharmaceutically acceptable carriers or excipients. Such pharmaceutical compositions may be prepared in a manner known in the art that is sufficiently storage stable and suitable for administration to humans and animals. The pharmaceutical composition may be lyophilised.

"Pharmaceutically acceptable" means a carrier or excipient that at the dosage and concentrations employed does not cause any unwanted effects in the patients to whom it is administered. Such pharmaceutically acceptable carriers or excipients are well-known in the art (see Remington's Pharmaceutical Sciences, 18th edition, A.R Gennaro, Ed., Mack Publishing Company (1990) and handbook of Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed ., Pharmaceutical Press (2000).

The pharmaceutical compostion may be admixed with adjuvants such as lactose, sucrose, starch powder, cellulose esters of alkanoic acids, stearic acid, talc, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulphuric acids, acacia, gelatin, sodium alginate, polyvinyl-pyrrolidine, and/or polyvinyl alcohol, and tableted or encapsulated for conventional administration. Alternatively, they may be dissolved in saline, water, polyethylene glycol, propylene glycol, ethanol, oils (such as corn oil, peanut oil, cottonseed oil or sesame oil), tragacanth gum, and/or various buffers. Other adjuvants and modes of administration are well known in the pharmaceutical art. The carrier or diluent may include time delay material, such as glyceryl monostearate or glyceryl distearate alone or with a wax, or other materials well known in the art.

The pharmaceutical compositions may be subjected to conventional pharmaceutical operations such as sterilisation and/or may contain conventional adjuvants such as preservatives, stabilisers, wetting agents, emulsifiers, buffers, fillers, etc., e.g., as disclosed elsewhere herein.

The pharmaceutical composition according to the invention may be administered locally or systemically such as topically, intravenously, orally, parenterally or as implants, and even rectal use is possible. Suitable solid or liquid pharmaceutal preparation forms are, for example granules, powders, tablets, coated tablets, (micro) capsules, suppositories, syrups, emulsions, suspensions, creams, aerosols, drops or injectable solution in ampule form and also preparations with protracted release of active compounds, in whose preparation excipients, diluents, adjuvants or carriers are customarily used as described above.

The pharmaceutical composition will be administered to a patient in a pharmaceutically effective dose. By "pharmaceutically effective dose" is meant a dose that is sufficient to produce the desired effects in relation to the condition for which it is administered. The exact dose is dependent on the, activity of the compound, manner of administration, nature and severity of the disorder, age and body weight of the patient different doses may be needed. The administration of the dose can be carried out both by single administration in the form of an individual dose unit or else several smaller dose units and also by multiple administration of subdivided doses at specific intervals. For example vaccination.

The pharmaceutical composition of the invention may be administered alone

or in combination with other therapeutic agents. These agents may be incorporated as part of the same pharmaceutical composition or may be administered separately.

The "patient" for the purposes of the present invention includes both humans and other mammal. Thus the methods are applicable to both human therapy and veterinary applications.

Following examples are intended to illustrate but not to limit the invention in any manner, shape, or form, either explicitly or implicitly.

EXAMPLES

Example 1

Production of methylation resistant plasmid DNA in bacteria.

1. Inocculate 2 ml conventional LB medium and 50 mikrogram/ml of ampicillin in a 10 ml test tube with an E.coli harbouring a plasmid of interest.
2. The culture is incubated in a shaker (200 rpm) over night at 37 °C.
3. The over night culture is then used to inoculate a 500 ml LB medium in a 2 liter culture flask. The flask is incubated at 37 °C in a shaker until the growth of the bacteria reaches OD(650) of 0,5 to 0,8.
4. 5'-deoxy-azacytidine or another deoxy-cytidine analogue is added to a concentration of 10 μM and chloramphenicol to a final concentration of 180 μg/ml is added.
5. The culture is incubated at 37 °C on a shaker as above over night.
6. The bacteria is pelleted by conventional centrifugation and plasmid DNA is isolated according to a standard Qiagen Inc. Maxiprep protocol.

Example 2

Production of adenovirus with methylation resistant DNA.

1. Plate 911 or 293 cells in conventional T-75 flasks to be 60-90% confluent at time of infection (about $1x10^7$ cells/T-75). Usually, fifteen to twenty T-75 flasks are sufficient to make a high titer stock.
2. Infect cells with virus supernatant at a multiplicity of infection (MOI) of 5 to 10 PFU (plaque forming units) per cell.
3. At day 2, add 5'-deoxy-azacytidine to a concentration of 10 μM.

4. When all cells have rounded up and about half of the cells are detatched (usually at 3 to 4 days post infection), harvest and combine all flasks. Spin 5 min in a benchtop centrifuge (~500 g), and remove the supernatant.
5. Resupend pellet in 8.0 ml sterile PBS. Perform four cycles of freeze/thaw/vortex. Centrifuge lysate in Sorvall HS4 rotor at 6000 rpm (7000 g) 4 °C for 5 min.
6. Weigh 4.4 grams of CsCl in a 50-ml conical tube, transfer 8.0 ml of clear virus supernatant to the tube, and mix well by vortexing. Transfer the CsCl solution (about 10 ml, density of 1.35 g/ml) to a 12 ml polyallomer tube for SW41 rotor. Overlay with 2 ml mineral oil. Prepare a balance tube. Spin the gradient in SW41 rotor at 32,000 rpm, 10 °C, for 18 to 24 hours.
7. Collect virus fraction (about 0.5 to 1.0 ml) with a 3cc syringe and an 18g needle. Insert needle through the tube from below. Mix with equal volume 2X Storage Buffer (2X Storage Buffer = 10mM Tris, pH 8.0, 100 mM NaCl, 0.1% BSA, and 50% glycerol, filter sterilized). Store virus stocks at –20 °C.
8. Check viral titer by GFP (preferred) of by plaque assays (see below) or by immunohistochemical staining, or simply read OD at 260 nm. To read OD, add 15 µl virus to 15 µl blank solution (Blank Solution = 1.35g/ml CsCl mixed with equal vol 2X Storage Buffer) plus 100 µl TE/0.1% SDS; vortex 30 seconds, centrifuge 5 min. measure A260. One A260 unit contains ~1 x 1012 viral particles (particles:infectious particles should be about 20:1).

Example 3

Measurement of the degree of 5-azadeoxycytidine incorporation in DNA.

Method 1

Add 5'-deoxy-azacytidine to the media of the growing cells at a concentration of 10 µM. Also add a trace amount ^{14}C-labelled deoxy-azacytidine, e.g., 5 µCi. If the specific activity of the labelled azacytidine is 500 Ci/mmol this is equivalent to 10 pmol of azacytidine. If the volume used is 100 ml, then the concentration of the radio-labelled nucleotide is 100 pM. The relation between the cold and labelled nucleotide is then 100.000:1. The radioactivity of the isolated DNA is measured in a scintillation counter and the incorporation is estimated dependant on the efficiency of the scintillation counter (1 Ci = 1 x 10^{10} dps).

Method 2

Add 5'-deoxy-azacytidine to the media of the growing cells at a concentration of 10 μM. The aza-cytdine molecule can be separated by means of HPLC from cytidine. The isolated DNA is treated with DNAse I so that a high concentration of free nucleotides is generated. The samples is injected in a suitable HPLC column and the nucleotides is separated into cytidine, thymidine, guanine, adenine and 5'azacytdine respectively. The concentration of aza-cytidine in relation to cytidine is measured.

Example 4

Production of vector DNA, in vitro, using PCR.

A transcriptional unit containing a CMV promoter, the gene encoding green fluorescent protein (GFP) and an HSV-TK polyadenylation site framed by two insulators in the opposite direction was cloned in a plasmid. The opposite directions of the two chicken beta globin insulators was used so that if recombination occurs, the orientation of the transcriptional unit was only inverted and not lost. The purpose is to amplify the transcriptional unit with base analogues that make it methylation resistant and to introduce the unit into human glioma cells. Different ratios between the base analogue 5'-aza-2'-deoxy cytidine triphosphate and 2'-deoxy cytidine triphosphate was used to generate vector DNA with different levels of methylation resistant 5'-aza cytidine.

1. 10 ng of the cloned gene construct in the shuttle vector pAd-easy was mixed in a reaction tube containing a buffer for pfu DNA polymerase with 5 mM of $MgCl_2$, 100 mM Tris at pH 7,4, deoxy-nucleotide triphosphate mixture containing 250 micromolar of dATP, dGTP and dTTP. The reaction mixture also contained a mixture between 5'-aza-2-deoxycytidine triphosphate and 2'-deoxycytidine triphosphate in the following ratios; 100:0, 75:25, 50:50, 25:75, and 0:100 so that the final concentration is 250 micromolar of the two cytidines together. The reaction mixture also contains two primers, froward primer is CAGGACTCTGATGGAACCAGG and the reverse primer is CCCTACCATTAGATGGATCAG. The reaction mixture contains 250 ng of each primer and the reaction volume is 50 microliter.

2. To the five different tubes, 2 units of pfu DNA polymerase (Stratagene Inc., USA) was added and 20 cycles of PCR are run with an annealing temperature of

56 °C for 1 minute, a reaction temperature of 72 °C for 2 minutes and a denaturation temperature of 95 °C for 40 seconds.

3. After PCR the five reactions were undertaken agarose gel electrophoresis and under mild UV, the five different bands were excised from the gel and the DNA of the five different fragments were isolated from the gel.

4. To three different primary human glioma cell cultures, 1 microgram of each of the five different vectors were used for transfection following the protocol from Boehringer & Mannheim using FUGENE. The cell number that was used for each transfection was 5×10^6 cells in ten different tissue culture flasks.

5. After 24 hours the cells were trypsinated and transferred to 10 new tissue culture flasks and 1×10^5 cells were used for FACS analysis, monitoring the transient expression from the ten different samples.

6. One week after transfection the cells were trypsinated again and the cells were sorted using the FACS (Becton Dickinson Inc., USA). The GFP positive cells were replated onto 24 well plates. The cultures were followed and monitored using a fluorescent inverted microscope (Olympus). Another two weeks after expanding the cultures the cells were once again subjected to FACS cell sorting.

7. The GFP positive cells were diluted to 0,5 cells per 100 microliter and 100 microliter were put into 96 replicate wells for each of the ten different cultures. One-cell clones that originated from this cloning were measured for long term expression of GFP.

Example 5

Constuction of a Transgenic Plant

Competent cells of Agrobacterium tumefaciens were subjected to transfection with the plasmid pRT101 in which the gene for green fluorescent protein were cloned in the Eco RI site of the plasmid and was under the control of the Cauliflower mosaic virus promoter (CaMV). The bacteria were grown in YM broth (0.04% yeast extract, 1% mannitol, 1.7 mM NaCl, 0.8 mM MgSO4·7H20, 2.2 mM K2HPO4·3H20, (pH 7.0)) and at and OD of 0,1 the medium was supplemented with

100 micromolar of 5'-aza-2'-deoxycytidine. The plasmid DNA was isolated using standard technique with a kit from Qiagene Inc.

The plasmid DNA isolated was used for electroporation of callus cells from barley (Hordeum vulgare). As a control the same plasmid not containing aza-cytidine was used.

The transformation frequency was followed under an inverted fluorescent microscope and stable clones from both natural plasmid transfection as well as those transformed with plasmids containing aza-cytidine were subjected to differentiation and plants were developed from both categories. The expression of the jelly-fish protein (GFP) over long term in the two groups were studied.

CLAIMS

1. A vector comprising a nucleotide sequence wherein one or two cytosines in at least one CpG motif has been replaced with a cytosine analogue resistant to methylation.
2. The vector according to claim 1, wherein the cytosine analogue replaces the 5-position in the pyrimidin ring of cytosine located in the CpG motif with N, O or C-X.
3. The vector according to claim 2, wherein cytosine analogue is a cytidine derivative.
4. The vector according to claim 3, wherein the cytidine derivative is 5-azacytidine and/or 5-azadeoxycytidine.
5. The vector according to claim 2, wherein X is a low or non-electrophilic group.
6. The vector according to claim 5, wherein the low non-electrophilic group is selected from the group consisting of ethyl and methoxy.
7. The vector according to any of the preceding claims, wherein the replaced cytosine or cytosines are located in one and the same DNA strand or in both DNA strands of the vector.
8. The vector according to any of the preceding claims, wherein the vector is a linear, circular, single or double stranded vector.
9. The vector according to any of the preceding claims, wherein the vector is selected from the group consisting of bacteriophages, plasmids, phagemids, viral vectors, plant transformation vectors, insect vectors and yeast artificial chromosomes.
10. The vector according to any of the preceding claims, wherein the number of cytosine analogues and the distribution of the cytosine analogues render the nucleotide sequences including said cytosine analogues resistant against methylation and maintain the expression of the nucleotide sequences including cytosine analogue containing CpG motifs.
11. The vector according to any of the preceding claims, wherein at least 1 % of the CpG motifs are replaced with cytosine analogues.
12. The vector according to claim 11, wherein at least 5 % of the CpG motifs are replaced with cytosine analogues.
13. The vector according to claim 12, wherein at least 10 % of the CpG motifs are replaced with cytosine analogues.
14. The vector according to claim 13, wherein at least from about 1% to about 100 % of the CpG motifs are replaced with a cytosine analogues.
15. The vector according to any of the preceding claims, wherein the vector comprises at least one gene or part of one gene.

16. The vector according to any of the preceding claims, wherein the vector comprises one or more expression control sequences.
17. A donor host cell comprising a vector according to any of claims 1-16.
18. The donor host cell according to claim 17, wherein the donor cell is an eukaryotic or a procaryotic cell.
19. The donor host cell according to claim 18, wherein the donor host cell is selected from the group consisting of an isolated bacteria, fungi, plant, insect, mammal, or other appropriate animal cells or cell lines, as well as transgenic animals or plants.
20. The donor host cell according to any of the claims 16-19, wherein the donor host cell is a host cell belonging to a GMP certified cell-line.
21. The donor host cell according to claim 20, wherein the cell-line is a mammalian cell-line.
22. A nucleotide sequence obtained from the donor host cell according to any of the claims 17-21, the nucleotide sequence being part of the vector according to any of the claims 1-16.
23. The nucleotide sequence according to claim 22, wherein the nucleotide sequence is one or more genes and/or part of one or more genes and/or one or more expression control sequences and/or a transcriptional unit.
24. A polypeptide produced by the donor host cell according to any of the claims 17-21, the nucleotide sequence encoding the polypeptide being part of the vector according to any of the claims 1-16.
25. A pharmaceutical composition comprising the vector according to any of the claims 1-16 and/or the donor host cell according to any of the claims 17-21 and/or the nucleotide sequence according to the claims 22-23 and/or the polypeptide according to the claim 24 and a pharmaceutically acceptable diluent, carrier, adjuvant or excipient.
26. A method of reducing methylation of CpG motifs in a vector in a receiver host, which method comprises replacing at least one cytosine in a CpG motif with a cytosine analogues according to any of the claims 1-16.
27. The method according to claim 26 wherein the method comprising the steps of i) providing a vector; ii) transferring the vector into a donor host cell; iii) growing the donor host cell harbouring the vector in a growth medium consisting of a suitable amount of cytosine analogues and iv) harvesting the multiplied vector comprising of incorporated cytosine analogues in at least one CpG motifs.
28. The method according to claim 27 wherein the cytosine analogue is a cytidine derivative
29. The method according to claim 28 wherein the cytidine derivative is 5-azacytidine and/or 5-azadeoxycytidine.

30. The method according to any of the claims 27-29 wherein the harvested vector is a vector according to any of the claims 1-16.
31. The method according to any of the claims 26-30, wherein the donor host cell is a donor host cell according to any of the claims 17-21.
32. The method according to any of the claims 26-31 used in small- or large-scale production of a nucleotide sequence and/or a polypeptide within the chemical industry such as the pharmaceutical industry.
33. A kit comprising a vector according to any of the claims 1-16 and/or a donor host cell according to any of the claims 17-21.
34. A method of transferring a vector according to any of the claims 1-16, a nucleotide sequence according to any of the claims 22-23, a polypeptide according to claim 24 or a pharmaceutical composition according to claim 25 into a receiver host cell, the method being selected from the list consisting of electroporation, microprojectile bombardment and liposome mediated delivery.
35. A vector according to any of the claims 1-16, a donor host cell according to claims 17-21, a nucleotide sequence according to any of the claims 22-23, a polypeptide according to claim 24 or a pharmaceutical composition according to claim 25 for use in therapy and/or in diagnostics
36. Use of a vector according to any of the claims 1-16, a donor host cell according to claims 17-21, a nucleotide sequence according to any of the claims 22-23, a polypepitde according to claim 24 or a pharmaceutical composition according to claim 25 for the manufacture of a medicament for use in therapy and/or in diagnostics.

Fig 1 /2

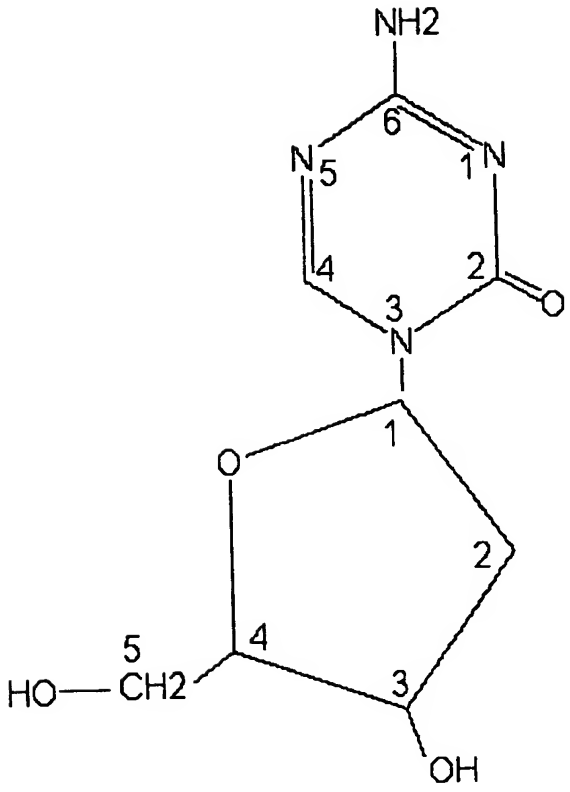
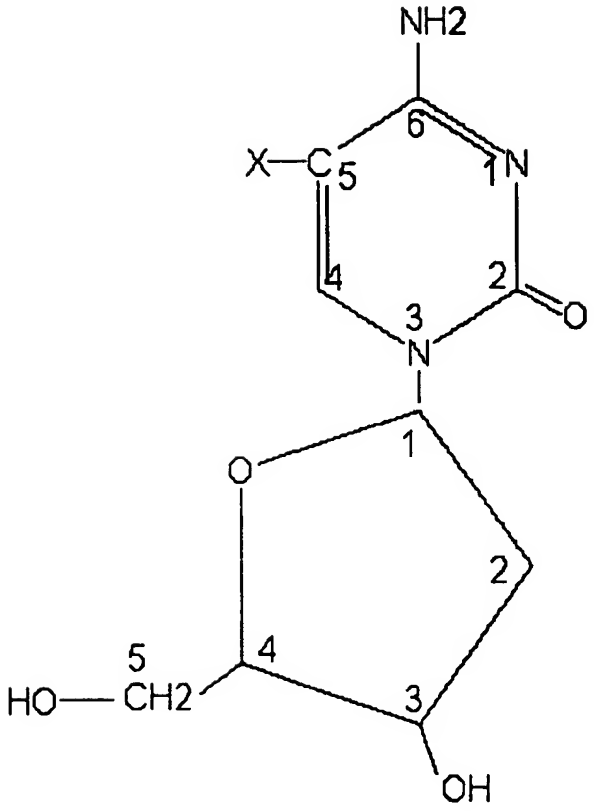


Fig 2/2



INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 02/01121

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12N 15/63
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12N, C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-INTERNAL, WPI DATA, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	KLAUDYNE, Hong et al. "Methylation of episomal plasmids as a barrier to transient gene expression via a synthetic delivery vector". BIOMOLECULAR ENGINEERING, 2001, Vol. 18, pages 185 - 192, abstract.	1-36
	--	
A	SANTI, Daniel V. et al. "Covalent bond formation between a DNA-cytosine methyltransferase and DNA containing 5-azacytosine". PROC. NATL. ACAD. SCI. USA, November 1984, Vol. 81, pages 6993 - 6997, see page 6993, column 1 - column 2, line 16.	1-36
	--	

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 November 2002	14 -11- 2002
Name and mailing address of the ISA/ Swedish Patent Office, Box 5055, S-102 42 STOCKHOLM, Facsimile No. +46 8 666 02 86	Authorized officer: Frida Plym Forshell /OGU, Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 02/01121

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JONES, Peter A. et al. "Cellular Differentiation, Cytidine Analogs and DNA Methylation". CELL, May 1980, Vol. 20, pages 85 - 93, abstract. --	1-36
A	BRODAY, Limor et al. "5-Azacytidine Induces Transgene Silencing by DNA Methylation in Chinese Hamster Cells". MOLECULAR AND CELLULAR BIOLOGY, April 1999, Vol. 19, No. 4, pages 3198 - 3204, see page 3198, column 2, line 37 - page 3199, column 1, line 9. --	1-36
A	SCHEULE, Ronald K. "The role of CpG motifs in immunostimulation and gene therapy" ADVANCED DRUG DELIVERY REVIEWS, 2000, Vol.44, pages 119 - 134, see page 121, column 2, "Cytosine methylation". --	1-36
A	HUG, Martin et al. "Transcriptional repression by methylation: cooperativity between a CpG cluster in the promoter and remote CpG-rich regions" FEBS Letters, 1996, Vol. 379, page 251 - 254, abstract. --	1-36
